

AD_____

Award Number: W81XWH-07-1-0134

TITLE: S-Nitrosylation and the Development of Pulmonary Hypertension

PRINCIPAL INVESTIGATOR: Lisa A. Palmer, Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, VA 22904

REPORT DATE: February 20F€

TYPE OF REPORT: Annual

PREPARED FOR: U. S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

X Approved for public release; distribution unlimited

Distribution limited to U. S. government agencies only;
Report contains proprietary information

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 14-FEB-2010		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 15 JAN 2009 - 14 JAN 2010	
4. TITLE AND SUBTITLE S-Nitrosylation and the Development of Pulmonary Hypertension				5a. CONTRACT NUMBER W81XWH-07-1-0134	
				5b. GRANT NUMBER PR064021	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Lisa A. Palmer Email: lap5w@virginia.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Virginia Charlottesville, VA 22904				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command Fort Detrick, Maryland 21702				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Nitric oxide (NO) transfer reactions between protein and peptide cysteines are thought to represent a regulated signaling process. In the pulmonary endothelium, endothelial nitric oxide synthase is required for the formation of S-nitrosothiols whereas, S-nitrosoglutathione reductase (GSNO-R) is involved in S-nitrosothiol breakdown. Interestingly, both proteins are regulated by sex steroids: eNOS activity is upregulated by estrogen and GSNO-R is downregulated by testosterone. Previous studies suggest that the differences in GSNO-R activity may be responsible for the gender dependent effects seen in our model of pulmonary hypertension. Examination of S-nitrosothiol hemoglobin content in blood taken from the right ventricle from male and female C57BL6 animals show no differences, suggesting in health, the activities of eNOS and GSNO-R are balanced. Molecularly, we have determined that these two proteins interact, either directly or indirectly and treatment with an S-nitrosylating agent disrupts this interaction. In addition, both eNOS and GSNO-R are S-nitrosylated and overexpression of GSNO-R in endothelial cell culture modifies phosphorylation of eNOS serine 1177, which has been implicated in eNOS activation. Pulmonary responses to eNOS knockout mice demonstrated elevated right ventricular pressures only with SNOAC and Hypoxia. GSNO-R deficient mice fail to respond to any treatment. Taken together, this suggests the existence of an S-nitrosylation/denitrosylation coupling loop and importance on the activity of GSNO-Reductase. Disruption of this loop may lead to the development of pulmonary disease.					
15. SUBJECT TERMS None provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT U	18. NUMBER OF PAGES 16	19a. NAME OF RESPONSIBLE PERSON Lisa A. Palmer
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code) (434) 924-2132

Introduction:

Pulmonary arterial hypertension (PAH), high blood pressure within the lung, is a progressive disease which is characterized by an increase in pulmonary arterial pressure and the formation of muscle around normally non-muscular small pulmonary arteries. Without treatment, PAH progresses rapidly to right heart failure and death. The objective of the current proposal is to define the role of S-nitrosothiols in the development and progression of PAH. To this end, we have previously developed a model in which N-acetyl cysteine (NAC) is used as a tracer to 1) monitor S-nitrosothiol (SNO) formation, transfer and metabolism *in vivo*, 2) address the physiological and pathological consequences of SNO signaling in the pulmonary vasculature, and 3) identify SNO target proteins in this signaling pathway. Differences in SNO formation, transfer and metabolism and the role this pathway plays in gender specific differences in the presentation of this disease are examined.

S-nitrosylation, a redox-based modification of a cysteine thiol by nitric oxide, is a post translational modification that can alter a protein's function. Mechanisms that control the addition or removal of the NO group from cysteine thiols are essential in determining the net effect of this modification. Formation of endogenous S-nitrosothiols can be mediated through 1) the activity of any one of the nitric oxide synthase (NOS) isoforms, 2) oxidative reactions generating nitrosative species (Fe^+NO , N_2O_3) or 3) transnitrosative reactions (NO^+ -transfer). The production of S-nitrosothiols is opposed by mechanisms mediating denitrosylation which can occur non-enzymatically via homolytic or heterolytic cleavage, catalyzed by transition metal ions and reactive oxygen species, or through enzymatic degradation. One specific enzyme that regulates S-nitrosothiol catabolism is S-nitrosogluthathione reductase (GSNO-R). Previous studies from this proposal suggested that one protein that may be involved in the gender-dependent preferences for disease presentation is GSNO-R. Thus, studies for this grant period (January 15, 2009 to January 14, 2010) have focused on the expression and activity of GSNO-R in the pulmonary endothelium and the influence of this protein in the gender predilection seen in this disease.

Body:

GSNO-R is a highly conserved, ubiquitously expressed NADP-dependent enzyme responsible for the breakdown of S-nitrosogluthathione (GSNO) to oxidized glutathione and ammonia (1, 2). GSNO-R functions as a homodimer of two identical subunits (3, 4). Each subunit has two domains: a coenzyme binding domain forming the subunit interface and a catalytic domain located at the ends of the dimer (3, 4). There are two zinc atoms in each of the subunits (3, 4). One zinc atom is contained within the active site while the other is involved in structural integrity. The primary substrate for GSNO-R is S-nitrosogluthathione (GSNO), however levels of other SNO-proteins are affected indirectly through altered transnitrosation equilibria with GSNO.

Dysregulation of S-nitrosylation is involved in the aetiology of numerous diseases which include those of the muscle, heart, and lung (5). Gender differences in the activity and/or expression of GSNO-R have been suggested. For instance, gastric activity of GSNO-R may be a component of the enhanced vulnerability of women to develop alcohol-related diseases (6). Likewise, gender differences seen in the LPS model of septic shock are eliminated in GSNO-R knockout mice (1). We have previously

shown that: 1) chronic administration of N-acetyl cysteine (NAC) elicits PAH indistinguishable from that induced by chronic hypoxia in male C57BL6/129SvEv mice (7). This effect of NAC is not seen in female C57BL6SvEv mice; 2) the level of S-nitrosylated N-acetyl cysteine (SNOAC) found in the plasma of female mice was significantly less than that seen in male mice, suggesting that altered catabolism of S-nitrosothiols may be responsible for this difference. The data presented examines the relationship of GSNO-R to gender and the influence this may have in the development of PAH.

GSNO-R activity is Greater in the lungs of Female mice.

The activity and expression of GSNO-R in lung homogenates from male and female mice were examined. GSNO-R activity was significantly higher in female animals compared to their corresponding male counterparts (Figure 1A). To determine if the differences in GSNO-R activity were due to changes in protein expression, Western blot analysis was performed (Figure 1B). No significant differences in GSNO-R protein expression in the lung were seen between male and female animals.

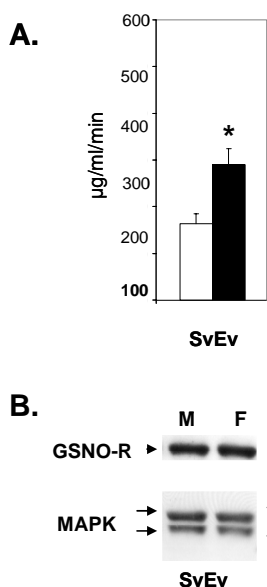


Figure 1. GSNO-R activity is greater in the lungs of female mice. A. GSNO-R activity is measured in male and female C57BL6SvEv (SvEV) mice. GSNO (35µM) was incubated with 500 ng/µl of lung homogenate supplemented with NADH (300 µM), GSH (2mM), ascorbic acid (500 µM), DTPA (100 µM) and PBS pH 7.4. After incubation at 37°C for 5 minutes the enzyme activity was quenched with trichloroacetic acid (5%) and GSNO determined by mass spectrometry. GSNO-R activity in the lung homogenates obtained from female mice was significantly **elevated** compared to male mice in the C57BL6/129SvEv (n=4, p<0.05). (B) Western blot demonstrating that protein expression of GSNO-R in the lung is similar in male and female mice. Equal loading of the samples was determined by MAPK.

GSNO-Reductase is an S-nitrosylated protein.

The detected difference in GSNO-R activity present in the lung homogenates of male and female animals could be explained by differences in S-nitrosylation. To examine if GSNO-R is an S-nitrosylated protein, lung homogenates obtained from male and female animals were subjected to biotin switch followed by Western blot analysis (Figure 2). GSNO-R was found to be S-nitrosylated in both male and female lung homogenates. The level of S-nitrosylation was greater in lung homogenates of female mice as compared to lung homogenates from male animals. S-nitrosylation was confirmed by the ability of mercuric chloride to significantly reduce the appearance of S-nitrosylated GSNO-R. As before, no differences in GSNO-R protein expression were seen in the lungs of male and female mice.

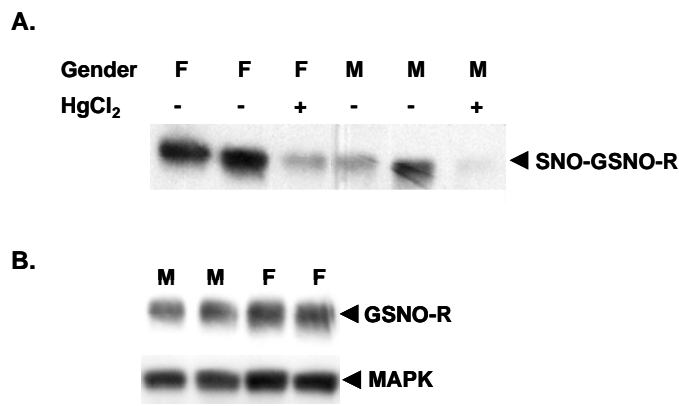


Figure 2. Female lungs have more S-nitrosylated GSNO-R than male lungs.

(A) Lung homogenates (200µg) from male and female animals were subjected to biotin-switch in the presence or absence of mercuric chloride then subjected to Western blot analysis. Lung homogenates obtained from female mice had more S-nitrosylated GSNO-R than that seen male animals. Mercuric chloride effectively eliminated the abundance of S-nitrosylated GSNO-R. (2) Western blot analysis of total GSNO-R protein in male and female lung homogenates.

S-nitrosylation activates GSNO-R. Post-translational modifications by S-nitrosylation often results in a change in a protein's activity. Previous data demonstrate lung homogenates obtained from female animals have greater GSNO-R activity (Figure 1) and elevated GSNO-R S-nitrosylation (Figure 2) compared to male animals, suggesting that S-nitrosylation and activity are related. To define the impact of S-nitrosylation on the activity of GSNO-R, murine pulmonary endothelial cells were treated with and without 10µM GSNO for 10 min. GSNO-R activity was measured in cell homogenates obtained from the untreated and treated cells. Treatment of murine lung endothelial cells with GSNO resulted in a significant increase in GSNO-R activity (Figure 3).

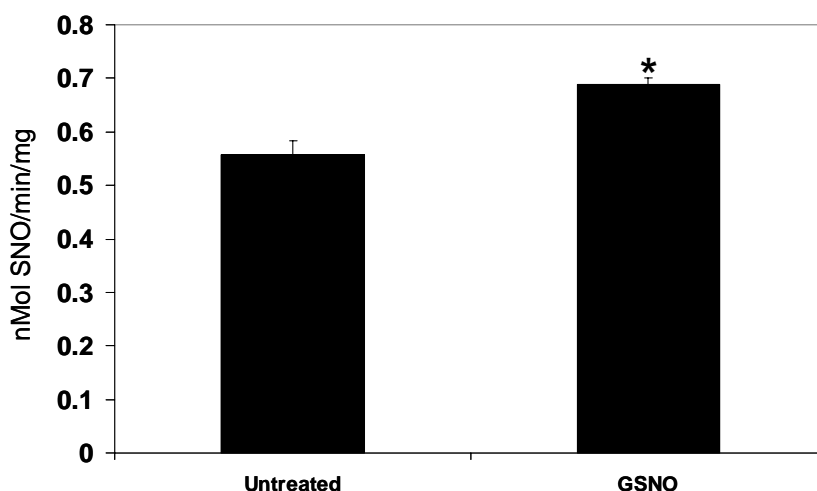


Figure 3. GSNO increases the activity of GSNO-R. GSNO-R activity was measured in cell lysates from murine pulmonary endothelial cells treated with and without 10µM GSNO by using a modified Saville Assay. Briefly, 250 µg of cell lysate from untreated and treated cells was incubated with 300µM NADH, 2mM GSH and 28µM GSNO. Two aliquots of 75µl were placed into a 96 well plate at 1 min intervals for a total of 5 min. One aliquot was placed with 75µl of (+) reagent (58 mM Sulfanilamide + 7.36 mM HgCl₂ in 1N HCl) while the second aliquot was placed with (-) reagent (58 mM Sulfanilamide in 1N HCl). Samples were incubated 5 min in the dark. At the end of this incubation, 75µl of (N) reagent (0.77M n-(1-naphthyl) ethylene-diamine dihydrochloride) was added. Samples were incubated 5-10 min for color to develop. Absorbance was read at 540nm. Amount of GSNO remaining in the reaction was determined from a GSNO

standard curve. Activity was obtained from the slope of the time course divided by the amount of protein in the reaction. ($p < 0.05$)

eNOS knockout mice have decreased GSNO-R activity.

The addition of exogenously added GSNO alters the activity of GSNO-R, suggesting that GSNO-R activity is altered by S-nitrosylation. To determine if modulation of S-nitrosothiols levels in vivo alters GSNO-R activity, we examined GSNO-R activity in the lungs of eNOS deficient (eNOS^{-/-}) mice (reduced S-nitrosothiol levels) and compared that to the activity in their strain matched controls (C57BL6) (Figure 4). The activity of GSNO-R in eNOS^{-/-} mice is significantly less than wild type mice, consistent with the idea that S-nitrosylation modifies GSNO-R activity.

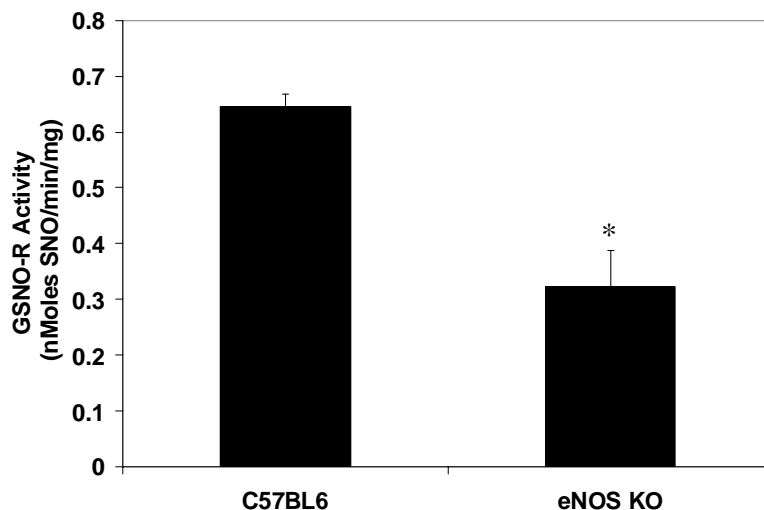


Figure 4. GSNO-R activity is reduced in eNOS^{-/-} mice. GSNO-R activity was determined in lung homogenates generated from male eNOS^{-/-} (eNOS KO) and strain matched control (C57BL6) mice as described in Figure 3. GSNO-R activity was significantly less in the eNOS^{-/-} animals. (n=5 C57BL6, N=6 eNOS^{-/-}, $p < 0.02$)

Identification of the Critical S-nitrosylation sites in GSNO-R.

Previous data demonstrates, for the first time, that GSNO-R is an S-nitrosylated protein. However, the cysteine residues important in GSNO-R S-nitrosylation are unknown. Inspection of the human GSNO-R sequence (Figure 5A) indicates the presence of a total of 15 cysteine residues (highlighted in green and red). To date, we have used site directed mutagenesis to generate cysteine to alanine mutations and have confirmed the sequence of five cysteine to alanine mutations (indicated in green). Mutations were generated at cysteines 8, 45, 174, 240 and 282 (Figure 5a, green residues). Initial studies examined the ability of these GSNO-R mutants to be S-nitrosylated. To date, we have examined the ability of C8, C240 and C282 of GSNO-R to be S-nitrosylated. Preliminary studies demonstrate that residues C240 and C282 are required for S-nitrosylation of GSNO-R.

A. Amino Acid Sequence of GSNO-R

MANEVIKCA AVAWEAGKPL SIEEIEVAPP KAHEVRIKII ATAVCHTDAY TLSGADPEGC
 FPVILGHEGA GIVESVGEGV TKLKAGDTVI PLYIPQCGEC KFCNPKTNL CQKIRVTQ GK
 GLMPDGTSRF TCKGKTILHY MGTSTFSEYT VVADISVAKI DPLAPLDKV LLGCGISTGY
 GAAVNTAKLE PGSVCAVFLG GGVGLAVIMG CKVAGASRII GVDINKDKFA RAKEFGATEC
 INPQDFSKPI QEVLIEMTDG GVDYSFECIG NVKVMRAALE ACHKGWGVS VVGVAASGEE
 IATRPFQLVT GRTWKGTAFG GWKSVESVPK LVSEYMSKKI KVDEFVTHNL SFDEINKAFE
 LMHSGKSIRT VVKI

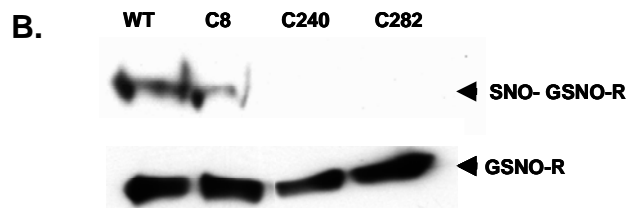


Figure 5. S-nitrosylation is abolished in GSNO-R mutants C240A and C282 A. Mouse lung endothelial cells were transfected with FLAG-tagged wild type (WT) GSNOR and GSNO-R cysteine to alanine mutants at residues 8, (C8), 240 (C240) and 282 (C282). Cell lysates were subjected to western blot analysis to ensure equal expression (GSNO-R) and to the biotin swith assay for the identification of S-nitrosothiol GSNO-R (SNO-GSNO-R) to determine S-nitrosylation status. S-nitrosylation of GSNO-R was only seen in WT and C8 transfected cells.

GSNO-R reduces eNOS phosphorylation at Serine 1177, a residue implicated in eNOS activation. The formation of S-nitrosothiols is mediated, at least in part by the activation of nitric oxide synthases (8-11). We have previously shown that eNOS interacts, either directly or indirectly with GSNO-R. In addition, eNOS has been shown to be an S-nitrosylated protein (12, 13). Moreover, when S-nitrosylated, eNOS is inactive (12, 13). To determine if GSNO-R has any effects on eNOS phosphorylation at 1177, a residue implicated in eNOS activation, murine pulmonary endothelial cells were transfected with human GSNO-R and the expression of phosphorylated and total eNOS examined in the absence of presence of increasing concentration of GSNO (Figure 6). Over-expression of GSNO-R resulted in a dramatic loss of eNOS phosphorylation at serine 1177. In addition, there was a slight, but insignificant decrease in total eNOS levels. Exogenous treatment with GSNO resulted in a dose dependent partial restoration of eNOS phosphorylation at serine 1177, demonstrating that GSNO-R is involved in the regulation of eNOS activity.

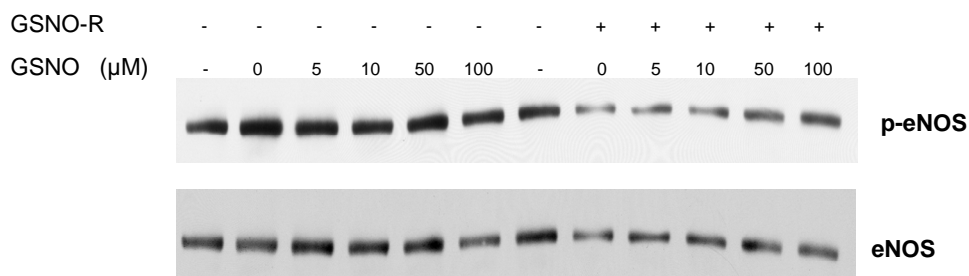


Figure 6. Overexpression of GSNO-R decreases phosphorylated eNOS. Murine pulmonary endothelial cells were transfected with GSNO-R and treated with increasing concentrations of GSNO. Whole cell lysates from transfected cells were subjected to Western blot analysis using antibodies against phosphorylated eNOS (peNOS, residue 1177) and eNOS (eNOS). Overexpression of GSNO-R significantly reduced peNOS expression as well as reduced total eNOS. Treatment with increasing concentrations of GSNO partially restored phosphorylated eNOS levels and had no significant effect on total eNOS levels.

Development of Pulmonary hypertension in GSNO-R knockout mice.

Imbalances in SNO bioavailability on the functioning of the pulmonary vasculature can be examined in vivo using GSNO-R deficient (decreased SNO turnover) mice. GSNO-R knockout (GSNO-R^{-/-}) breeding pairs were originally obtained from Jonathan Stamler (Duke University,1). Strain matched control animals are C57BL6 and are obtained from Jackson Laboratories. This year we were successful in breeding these animals and the studies evaluating the development of PAH in response to NAC, SNOAC, and hypoxia have been completed. GSNO-R^{-/-} animals demonstrated no significant gender specific differences in right ventricular weight (Figure 7A) or right ventricular pressure (Figure 7B) in response to NAC or SNOAC. Both male and female GSNO-R^{-/-} animals responded to hypoxia with increases in right ventricular weight and right ventricular pressure. Similar responses were seen with the GSNO-R^{+/-} animals in respect to changes in right ventricular weight. However, there were no significant differences seen

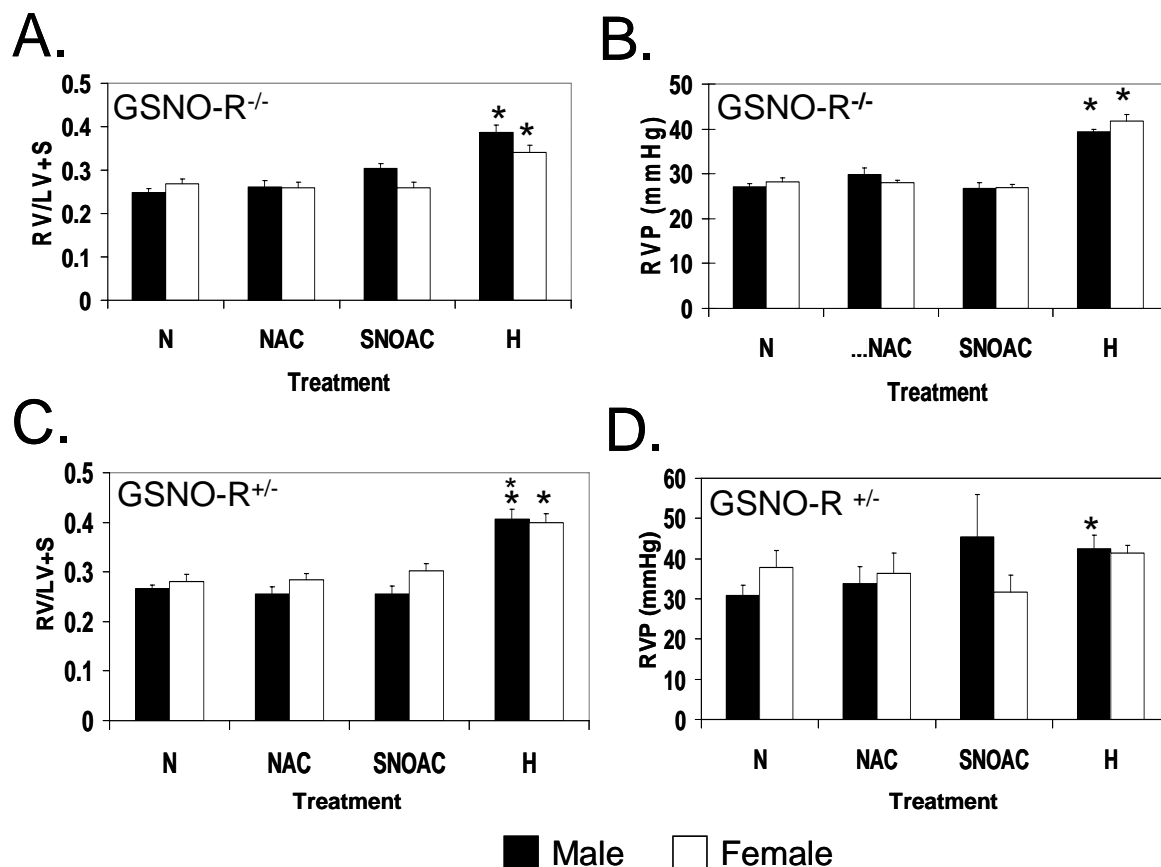


Figure 7. Development of Pulmonary Arterial Hypertension in Male and Female GSNO-R deficient (GSNO-R^{+/-}) and GSNO-R null (GSNO-R^{-/-}) mice. GSNO-R null (panels A, B) and GSNO-R deficient (panels C, D) were treated with NAC, or SNOAC or exposed to hypoxia for a period of 3 weeks. At the end of the exposure period, right ventricular weight and right ventricular pressure was measured as previously described (7). Black bars: males; white bars; females.

in the GSNO-R^{+/-} animals with respect to NAC, SNOAC, or hypoxia, in contrast to what was seen with the GSNO-R^{-/-} animals. The reason for this observation is not clear. One potential explanation for this difference may be due to complete compensation by other enzyme systems such as carbonyl reductase (14) or the thioredoxin/thioredoxin reductase (15) systems in the GSNO-R^{-/-} animals but not in the GSNO-R^{+/-} animals. Current studies are examining this possibility. Alternatively, these mice are a global knockout, thus the increase in SNO-protein levels may also alter other proteins involved in this response.

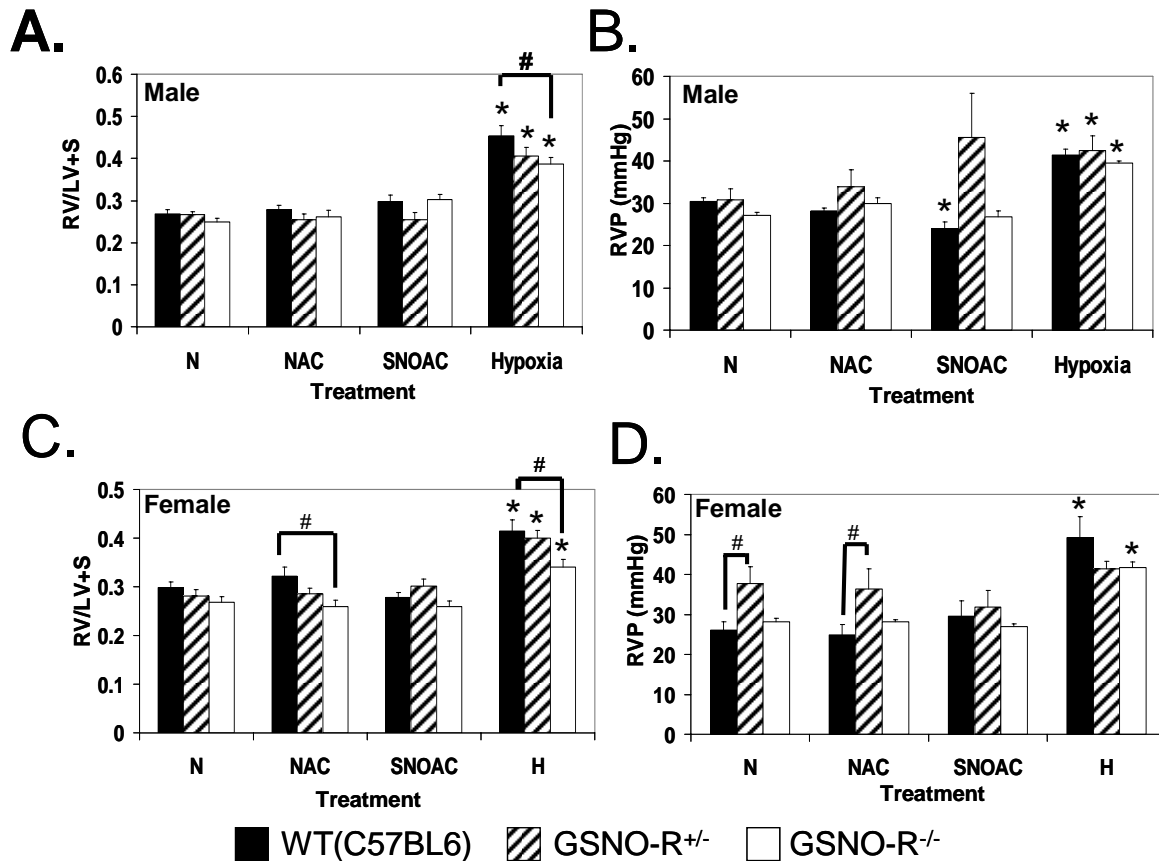


Figure 8. Effect of GSNO-R Gene Copy Number on Right Ventricular Weight and Right Ventricular Hypoxia. Male and Female wild type, GSNO-R^{+/-} and GSNO-R^{-/-} mice were exposed to 10mg/ml NAC, 52mM SNOAC and 10% hypoxia for three weeks as previously described (7). Right ventricular weight (Panels A, C) and right ventricular

pressures (Panels B, D) were measured as previously described (7). *, $p < 0.05$ compared to normoxic control; # $p < 0.05$ compared to wild type same condition.

We also compared the data obtained from the GSNO-R^{+/-} and GSNO-R^{-/-} animals to the strain matched wild type (C57Bl6) control animals in response to NAC, SNOAC and hypoxia (Figure 8). In male animals, there were no significant differences detected at baseline in right heart weight. Nor were there any significant differences noted in the magnitude of the response mediated by NAC and SNOAC. All animals, wild type, GSNO-R^{+/-} and GSNO-R^{-/-}, responded to hypoxia with increases in right heart weight. Significant differences in the magnitude of the response were seen in right heart hypertrophy with hypoxic exposure in male GSNO-R^{-/-} animals compared to the wild type controls, suggesting that loss of GSNO-R was protective. Decreases in right ventricular weight were also seen in GSNO-R^{+/-} animals compared to the wild type control animals, but these were not significant. Changes in right ventricular pressures were less clear. Basal pressures were not significantly different between wild type and GSNO-R^{+/-} animals, but were significantly reduced in the GSNO-R^{-/-} animals. Thus, loss of GSNO-R has influence on basal pulmonary vascular tone. NAC treatment had no effect in any of the animals. In contrast, treatment with SNOAC resulted in a decrease in right ventricular pressure in the wild type animals which was not seen in either GSNO-R^{+/-} or GSNO-R^{-/-} animals. Lastly, hypoxia resulted in an increase in right ventricular pressure for all genotypes. Moreover, all genotypes appeared to respond similarly.

In female animals, right heart weight appeared to be reduced with GSNO-R gene copy number compared to wild type animals, but this was not significant. In addition, there was no significant change in right heart hypertrophy with NAC in GSNO-R^{+/-} animals when compared to wild type animals. The magnitude of the changes was however, noted with the GSNO-R^{-/-} animals. There were no differences between genotype in response to SNOAC. All female animals showed an increase in right heart weight with hypoxia. However, the magnitude of the increase was significantly less in GSNO-R^{-/-} animals. Again, like the male animals, this would suggest that GSNO-R is protective. Right ventricular pressure in female animals appears to have much more variability than that seen in the male animals. There were no significant differences in right ventricular pressures seen at baseline, NAC or SNOAC when comparing wild type and GSNO-R^{+/-} animals. It should be noted that GSNO-R^{+/-} animals had baseline right ventricular pressures that were elevated compared to the wild type control animals. This difference was maintained in NAC. No significant differences were seen with SNOAC. Increases in right ventricular pressure were seen with the wild type and the GSNO-R^{-/-} animals with hypoxia. Although right ventricular pressures were all similar for the three genotypes, only the GSNO-R^{+/-} animals did not respond to hypoxia.

Endothelial/Erythrocyte Fibercell System:

An in vitro model for erythrocyte/endothelial cell interactions using Fibercell technology is currently being performed in the laboratory. The idea is that erythrocytes are a source of S-nitrosothiols which are in contact with the endothelium. S-nitrosothiols, through the process of transnitrosation will be transferred from the erythrocyte upon

deoxygenation to proteins in and on the endothelial cells. Hemoglobin is the key component of the erythrocyte involved in this process. It is known that S-nitrosothiol release from Hb is a function of oxygen saturation (16-20). In addition, binding of O₂ to Hb causes a change in its conformation, resulting in the transfer of nitrosonium (NO⁺) equivalents from the heme (Fe²⁺) to the thiol (cysteine β93) (17, 20, 21). Upon deoxygenation, NO⁺ equivalents are transferred from cysteine β93 to a receptor cysteine in anion exchange protein 1 (AE1) (22) on the erythrocytic membrane. AE1 exports NO⁺ equivalents through an unknown process thought to involve either direct cell-cell transfer or through the transnitrosation of glutathione or cysteine into S-nitrosoglutathione (GSNO) or S-nitrosocysteine (CysNO) respectively (1, 2, 7, 19, 23). At this time, it is not known what the target proteins are in and on the endothelial cells. The experiments proposed were designed to define some of these proteins.

Experiments performed in this funding period focused on the collection of S-nitrosylated endothelial cell proteins after exposure to the erythrocyte under normoxic, hypoxic, S-nitrosothiol depleted, and S-nitrosothiol loaded conditions. All samples were subjected to in vitro biotin switch to stabilize the S-nitrosylated endothelial proteins post exposure. To date, we have collected four samples, one sample from each of the four conditions identified above. All samples were collected from erythrocytes obtained from female donors. Progress in this project has been hampered by the low endothelial cell protein yields after removal from the fibercell system and by the susceptibility of the fibercell system to be contaminated with yeast. Low protein yields were remedied by increasing the number of fibercell cartridges per condition. It now appears that we will need at least 4 fibercell cartridges per each condition which will be pooled to obtain a single sample. Yeast contamination of the cultures appears to be seasonal, with spring and fall being the worst times. Additional care in the transfer of the cartridges in and out of the tissue culture hood and excessive washing of the stopcocks with ethanol have helped to minimize this problem. All samples are currently stored in the -80°C freezer until analysis. Analysis will be performed once three replicates for each condition are obtained.

Key Research Accomplishments:

Specific Aim 1: Aberrant formation/transfer and/or delivery of S-nitrosothiols (SNOs) leads to the development of Pulmonary Hypertension

Task 3: Identify the pathophysiological changes caused by NAC/SNOAC treatment in the pulmonary vasculature in vivo.

- a. Measure physiological parameters (changes in right heart weight, right ventricular pressure in wild type mice and GSNO-R^{+/-} and GSNO-R^{-/-} mice. (Figure 8 in text).
- b. Set up physiological relevant in vitro endothelial culture system using “fibercell technology” to establish normal flow mediated shear stress

- i. Collected endothelial protein samples from fibroblast system under normoxic, hypoxic, S-nitrosothiol loaded and S-nitrosothiol depleted erythrocyte conditions.

Specific Aim 3. Gender differences in pulmonary hypertension arise from an imbalance of SNO formation and metabolism.

- a. Compare the ability of NAC, SNOAC and hypoxia to induce pulmonary arterial hypertension in male and female GSNO-R^{+/-} and GSNO-R^{-/-} animals. (Figure 7)
- b. Identify differences in SNO formation, transfer and metabolism between male and female animals.
 - i. GSNO-R activity is greater in the lungs of female mice (Figure 1)
 - ii. Activity of GSNO-R in the lung is not due to gender dependent increases in protein expression (Figure 2).
 - iii. Identified that GSNO-R is an S-nitrosylated protein (Figure 3).
 - iv. Activation of eNOS is important for S-nitrosylation of GSNO-R (Figure 4)
 - v. GSNO-R influences the phosphorylation of eNOS at a residue important for eNOS activity. (Figure 6)
 - vi. Identified two residues in GSNO-R that are important for GSNO-R S-nitrosylation (Figure 5)

Reportable Outcomes.

Manuscripts:

1. Increased S-nitrosogluthione reductase activity in the female murine pulmonary vasculature. In preparation; estimated time of submission February 28th 2010. American Journal of Respiratory Cell and Molecular Biology.
2. Endothelial Nitric Oxide Synthase/S-nitrosogluthione Crosstalk: Identification of a feedback system that contributes to Nitric Oxide Bioactivity. In preparation; estimated time of submission May/June 2010. Journal of Biological Chemistry.

Abstracts:

1. Lewis SJ, May W, Young A, Yeman S, Palmer LA. Gender differences in the ventilatory Response to hypoxia. Abstract 6885 submitted to American Thoracic Society International Conference to be held in New Orleans, May 2010.
2. Brown Steinke K, de Ronde K, Sullivan S, Yeman S, Palmer LA. S-nitrosogluthione reductase and its role in pulmonary arterial hypertension. Abstract 6982 submitted to American Thoracic Society International Conference to be held in New Orleans, May 2010.
3. Yeman S, Marozkina NV, Palmer LA, Gaston B. Therapeutic ultrasound decreases mean right ventricular pressure in hypoxic mice submitted to American Thoracic Society International Conference to be held in New Orleans, May 2010.

4. Kavoussi PK, Ellen JH, Oliver JL, Woodson RI, Costabile RA, Steers WD, Palmer LA, Lysiak JJ ENOS S-nitrosylation and Erectile Function submitted to American Urological Association

New Grant applications:

- 1.) NIH RO1 S-nitrosylation in the Regulation of the Erectile Response
- 2.) AHA Grant in Aid: S-nitrosylation and erectile function
- 3.) Program Project Grant: Cellular S-nitrosothiol Signaling in Respiratory Biology: PI Project 2: S-nitrosothiol signaling in Pulmonary endothelial cells
- 4.) LaunchPad : Diabetes and Erectile Dysfunction
- 5.) Commonwealth Health Research Board: Polarized eNOS at myoendothelial junctions regulates development of erectile dysfunction.

It should be noted that the new grants on NO bioactivity and erectile dysfunction and the myoendothelial junction are direct extensions of the results obtained on endothelial nitric oxide synthase and S-nitrosoglutatione reductase interaction in the pulmonary endothelium.

Conclusions

In the pulmonary vascular bed, evidence has emerged for the following paradigm: Increased delivery of S-nitrosothiols to the pulmonary vascular bed results in activation of hypoxia-related gene regulatory proteins including specificity proteins and hypoxia-inducible factors (7). The downstream effect of the activation of these pathways results in the upregulation of hypoxia-regulated genes which leads to cellular and morphologic changes characteristic of pulmonary arterial hypertension. We believe that the excessive S-nitrosothiol release into the pulmonary vascular bed mimics hypoxia. This may provide insight, in part, to pulmonary arterial hypertension of diverse causes: 1) hypoxia itself results in transnitrosation from deoxyhemoglobin to glutathione and other thiols in the systemic periphery which then returns in erythrocytes and plasma to be released into the pulmonary vascular bed; 2) increases in venous return in high flow states increase the burden of S-nitrosothiols “dumped” to the lungs, possibly explaining high flow state induced pulmonary arterial hypertension; and 3) systemic inflammation can increase erythrocytic S-nitrosothiol load, contributing to the risk of pulmonary arterial hypertension in systemic inflammation.

Excessive S-nitrosylation in the pulmonary vascular endothelium causes, paradoxically, eNOS-dependent pulmonary arterial hypertension that is hypoxia mimetic in male mice (7). Components (eNOS expression and GSNO-R activity) necessary in the formation and metabolism of endogenously produced S-nitrosothiols are generally greater in females than in males. Endothelial NOS opposes the cellular effects of GSNO-R: eNOS promotes protein S-nitrosylation whereas, GSNO-R promotes protein de-nitrosylation. Estrogen causes a post genomic increase in eNOS expression (24, 25). In contrast, we have shown androgens to have a negative regulatory effect of GSNO-R activity. Thus, the increased eNOS activity in the female mouse lung might lead to the increased GSNO-R activity observed in female mouse lungs to counter-act the increased nitrosative stress associated with the increased eNOS activity. This activity, however,

was not determined by a difference in protein expression but rather by a difference in activity. To this effect, we have demonstrated that GSNO-R activity is increased in the female lung and GSNO increases GSNO-R activity through S-nitrosylation. Moreover, eNOS^{-/-} mice have decreased GSNO-R activity. Taken together, the data suggest that active eNOS may S-nitrosylate GSNO-R, activating it as a count-regulatory mechanism.

If increased GSNO-R activity in female mice can protect against the adverse effects of nitrosative stress associated with eNOS activation, chronic inflammation, chronic hypoxemia and high flow states, why are female humans at higher risk for the development of pulmonary arterial hypertension from a variety of different causes than are males. GSNO-R may be the answer to this question. In the absence of androgens, which we have previously shown to negatively regulate GSNO-R activity, and in the presence of activated eNOS, increased GSNO-R activity is protective. However, this system may leave females vulnerable to dysfunction in GSNO-R. For example, if there is decreased GSNO-R activation by eNOS or an androgen-mimetic effect to decrease GSNO-R activity in the female pulmonary vascular endothelium or elsewhere, by virtue of increased eNOS activity and chronic nitrosative stress, there will be an increased risk for hypoxia mimetic pulmonary vascular remodeling. There are single nucleotide polymorphisms that appear to be associated with both increased and decreased asthma risk by virtue of increased and decreased GSNO-R activity respectively (26). These data suggest that future studies regarding GSNO-R expression, genetics and activity in the lungs of women with pulmonary arterial hypertension may be worthwhile. In addition, this may be particularly important in protecting against the high flow states and chronic eNOS upregulation associated with mammalian pregnancy (27,28) and may help to explain why females in general are more tolerant of high altitude living than males (29,30).

References

1. Liu L, Yan Y, Zeng M., Zhang J., Hanes M A, Ahearn G, McMahon T J, Dickfeld T, Marshall HE, Que L. G, and Stamler JS. Essential role of S-nitrosothiols in vascular homeostasis and endotoxic shock. *Cell* 116: 617-628, 2004.
2. Liu L, Hausladen A, Zeng M, Que L, Heitman J, and Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 410: 490-494, 2001.
3. Sanghani PC, Robinson H, Bosron W F, Hurley TD. Human glutathione-dependent dehydrogenase. Structures of Apo, Binary, and Inhibitory ternary complexes. *Biochemistry* 41: 10778-10786, 2002.
4. Sanghani PC, Robinson H, Bennett-Lovsey R, Hurley TD, Bosron WF. Structure-function relationships in human Class III alcohol dehydrogenase (formaldehyde dehydrogenase). *Chemico-Biological Interactions* 143-44: 195-200, 2003.
5. Benhar M, Forrester MT, Stamler JS. Protein denitrosylation: enzymatic mechanisms and cellular functions. *Nature Reviews* 10: 721-732, 2009.
6. Baraona E, Abittan CS, Dohmen K, Moretti M, Pozzato G, Chayes ZW, Schaefer C, Lieber CS. Gender differences in pharmacokinetics of alcohol. *Alcohol Clin Exp Res* 25: 502-7, 2001.

7. Palmer LA, Doctor A, Chhabra P, Sheram ML, Laubach VE, Karlinsey MZ, Forbes MS, Macdonald T, and Gaston B. S-Nitrosothiols signal hypoxia-mimetic vascular pathology. *J. Clin. Invest.*, 117: 2592-2601 2007.
8. . Gow, A. J., Chen, Q., Hess, B. J., Day, H., Ischiropoulos, H., and Stamler J. S. Basal and stimulated protein S-nitrosylation in multiple cell types and tissues. *J. Biol. Chem.* 277: 9637-9640, 2002.
9. Mayer, B, Pfeiffer, S, Schrammel, A., Koesling, D., Schmidt, K., and Brunner, F. A new pathway of nitric oxide/cyclic GMP signaling involving S-nitrosoglutathione *J. Biol. Chem.* 273: 3264-3270, 1998.
10. Jaffrey SR, Erdjument-Bromage H, Ferris CD Tempst P, Snyder SH. Protein S-nitrosylation: a physiological signal for neuronal nitric oxide. *Nature Cell Biol* 3: 193-97, 2001.
11. Eu JP, Liu L, Zeng M, Stamler JS. An apoptotic model for nitrosative stress. *Biochemistry* 39: 1040-1047, 2000.
12. Erwin PA, Lin AJ, Golan DE, Michel T. Receptor-regulated dynamic S-nitrosylation of endothelial nitric-oxide synthase in vascular endothelial cells. *J. Biol Chem.* 280: 19888-19894, 2005.
13. Ravi K, Brennan LA, Levic S, Ross PA, Black SM. S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proc Natl. Acad Sci USA* 101: 2619-2624, 2004.
14. Bateman RL, Rauh D, Tavshanjian B, Shokat KM. Human Carbonyl Reductase 1 is an S-nitrosoglutathione Reductase. *J. Biol Chem.* 283: 35756-35762 2009.
15. Trujillo M, Alvarez M, Peluffo G, Freeman B, and Radi R. Xanthine oxidase-mediated decomposition of S-nitrosothiols. *J. Biol. Chem.* 273: 7929-7934, 1998.
16. Singel DJ and Stamler JS. Chemical Physiology of blood flow regulation by red blood cells: The role of nitric oxide and S-nitrosohemoglobin. *Annu. Rev. Physiol.* 67: 99-145, 2005.
17. Stamler JS, Jia L, Eu JP, McMahon TJ, Denchendo IT, Bonaventura J, Gernert K, Piantadosi CA Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 276: 2034-2037, 1997.
18. Sonveaux P, Lobysheva II, Feron O, McMahon TJ. Transport and peripheral bioactivities of nitrogen oxides carried by red blood cell hemoglobin: role in oxygen delivery *Physiology.* 22:97-112, 2007.
19. Doctor A, Platt R, Sheram ML, Eischeid A, McMahon T, Maxey T, Doherty J, Axelrod M, Kline J, Gurka, M, Gow, A, and Gaston B. Hemoglobin conformation couples erythrocyte S-nitrosothiol content to O₂ gradients. *Proc. Nat. Acad. Sci. USA* 102:5709-5714, 2005.
20. Luchsinger BP, Rich EN, Gow AJ, Williams EM, Stamler JS, Singel DJ. NO interaction with oxidized hemes in human hemoglobin: routes to SNO-hemoglobin formation with preferential reactivity within the β subunits. *Proc. Natl. Acad Sci USA* 100:461-466, 2003.
21. Pezacki JP, Ship NJ, Kluger R. Release of nitric oxide from S-nitrosohemoglobin. Electron transfer as a response to deoxygenation. *J. Am Chem. Soc.* 123:4615-4616, 2001.
22. Pawloski JR, Hess DT, Stamler. Export of red blood cells of nitric oxide bioactivity. *Nature* 409:622-626, 2001.

23. Lipton A, Johnson M, Macdonald T, Lieberman M, Gozal D, Gaston B. S-nitrosothiols signal the ventilatory response to hypoxia. *Nature* 413: 171-174, 2001.
24. Chen Z, Yuhanna IS, Galcheva-Bargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates nongenomic activation of eNOS by estrogen. *J. Clin Invest.* 103: 401-6, 1999.
25. Goetz RM, Thatte HS, Prabhakar P, Cho MR, Michel T, Golan DE. Estradiol induces the calcium-dependent translocation of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 96: 2788-2793, 1999.
26. Wu, H, Romieu I, Sienra-Monge J-J, del Rio-Nararro BE,, Anderson DM, Jenchura CA, Li H, Ramirez-Aguilar M, de Carment Lara-Sanchez I, London SJ. Genetic variation in S-nitroglutathione reductase (GSNOR) in childhood asthma. *J Allergy Clin Immunol* 120: 322-8, 2007.
27. Gaston B, Fry, E, Sears S, Heroman WM, Ignarro L, Stamler JS. Umbilical arterial S-nitrosothiols in stressed newborns: Role in perinatal circulatory transition. *Biochemical and Biophysical Research Communications.* 253: 899-901, 1998.
28. Funai, EF, Davidson A, Seligman SP, Finley TH. S-nitrosohemoglobin in the fetal circulation may represent a cycle for blood pressure regulation. *Biochemical and Biophysical Research Communications* 239: 875-877, 1997.
29. Beall CM, Song K, Elston RC, Goldstein MC. Higher offspring survival among Tibetan women with high oxygen saturation genotypes residing at 4,000m [Multicenter Study] *Proc Natl Acad Sci USA* 101: 14300-14304, 2004.
30. Beall CM Decker MJ, Brittenham GM, Kushner I, Gebremedhin A, Strohl KP, An Ethiopian pattern of human adaptation to high-altitude hypoxia. *Proc Natl Acad Sci USA* 99: 17215-17218, 2002.